patients suffering clinically significant anaemia, which we defined as a decline in Hb of more than 3 g/dL or <10 g/dL, which is the threshold at which RBV dose reduction is recommended according to the package insert, during the initial 12 weeks of treatment. Additionally, we examined the factors for Hb < 10 g/dL during the initial 12 weeks of treatment in logistic regression models that included gender, age, baseline weight, Hb, platelet count and *ITPA* genetic variants and so on as covariates.

Association between ITPA genetic variants and anti-HCV treatment outcomes or RBV adherence

We examined the association between *ITPA* genetic variants and anti-HCV treatment outcomes in logistic regression models that included gender, age, baseline Hb, platelet count, HCV RNA level and *IL28B* genetic variants as covariates. In addition, we analysed RBV adherence during treatment in these patients. The patients were divided into three groups by administered dosage of RBV: <60%, ≥60 to <80%, ≥80% of the planned RBV dosage.

Statistical analysis

Categorical variables were compared between groups by the chi-squared test or Fisher's exact test, and noncategorical variables, by the Student's t-test. Multivariate logistic regression analysis with stepwise forward selection was performed with $P < 0.05$ in univariate analysis as the criteria for model inclusion. $P < 0.05$ was considered significant. These statistical analyses were conducted using SPSS software package, version 18J (Chicago, IL, the USA).

RESULTS

Patient characteristics and distribution of ITPA and IL28B genetic variants

The clinical characteristics of the study population are described in Table 1. Genotyping of rs1127354 revealed that 224 patients (72%) had CC and 85 patients (28%) had CA or AA (CA/AA). *IL28B* genotype resistant to PEG-IFN and RBV, TG or GG (TG/GG) at rs8099917 was possessed by 33% (102/309) of the patients. There were no significant differences in baseline clinical characteristics between the patients with CC and CA/AA genotypes at rs1127354.

Association between ITPA genetic variants and Hb levels during PEG-IFN plus RBV treatment

Hb levels during the initial 12 weeks of therapy are shown in Fig. S1. Patients with CA/AA at rs1127354 showed a lower degree of Hb reduction at weeks 2, 4, 8 and 12 during therapy than those with CC ($P < 0.0001$ for weeks 2, 4, 8 and 12). The greatest difference in mean Hb reduction was found at week 4, −0.8 ± 0.9 g/dL in patients with CA/AA and −2.5 ± 1.2 g/dL in those with CC.

Table 1 Clinical characteristics of total 309 chronic hepatitis C patients

Characteristic	(n = 309)
Male gender	160 (52%)
Age, years	57 ± 10
Body weight, kg	60 ± 11
Haemoglobin, g/dL	14.1 ± 1.4
Platelet count, $\times 10^4/\mu L$	16.4 ± 5.6
ALT, IU/L	74 ± 65
γ-GTP, IU/L	61 ± 71
Creatinine, mg/dL	0.7 ± 0.2
HCV RNA, log IU/mL	6.2 ± 0.6
rs8099917, TT/TG+GG	207/102
rs1127354, CC/CA+AA	224/85

ALT, alanine aminotransaminase; γ-GTP, γ-glutamyl transpeptidase. rs8099917: TT is favourable to treatment efficacy, and rs1127354: CA/AA is protectable to RBV-induced anaemia in PEG-IFN plus RBV therapy.

Incidence of severe anaemia during PEG-IFN plus RBV treatment

As depicted in Fig. 1, the incidence of severe anaemia up to week 12 (≥3 g/dL reduction or <10 g/dL of Hb) was more frequent in the patients with CC at rs1127354 [65% (145/224), 33% (73/224)] than in those with CA/AA [25% (21/85), 6% (8/85)] ($P < 0.0001$). These results show that the CA/AA genotypes are significantly associated with less absolute reduction in Hb levels, especially during the early weeks of therapy, and protect against the development of severe anaemia.

Factors for the incidence of Hb < 10 g/dL up to week 12

By univariate analysis, pretreatment factors for severe anaemia, Hb < 10 g/dL up to week 12, were female gender, older age, lower body weight and baseline Hb level as well as rs1127354 CC genotype. Multivariate logistic regression analysis was then carried out using these factors as covariates. As a result, *ITPA* genotype, pretreatment Hb levels and age were independent predictive factors for severe anaemia (Hb < 10 g/dL) up to week 12 (Table 2). Therefore, we analysed the proportions of Hb < 10 g/dL up to week 12 by *ITPA* genotype, pretreatment Hb levels and age. As depicted in Fig. S2, the incidence of Hb < 10 g/dL up to week 12 was most frequent in ≥60-year-old patients with CC genotype of rs1127354 and baseline Hb < 14 g/dL [61% (39/64)], indicating that these patients were the highest risk group for severe anaemia.

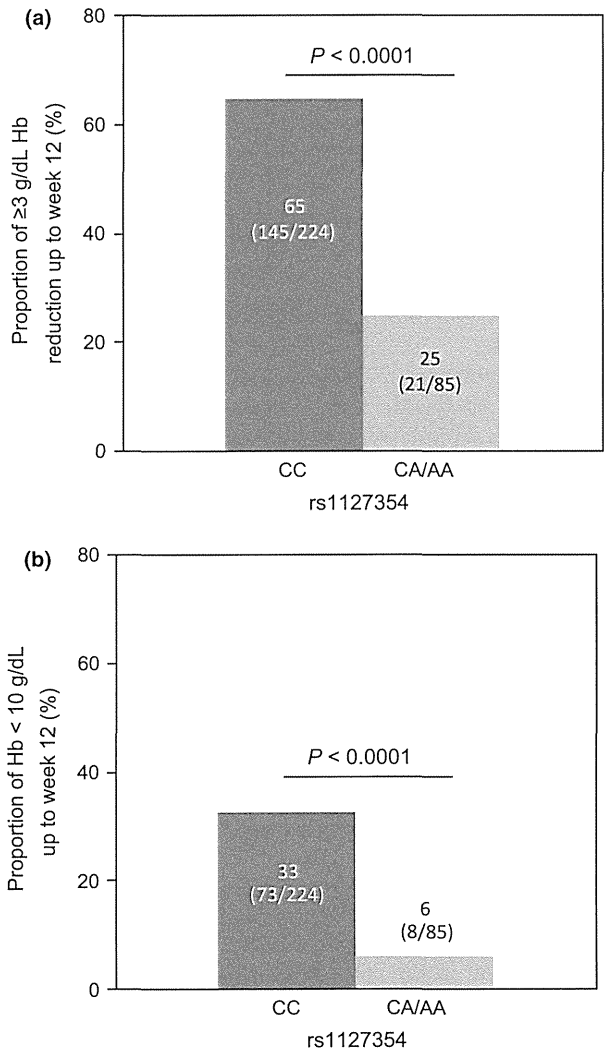

Fig. 1 Proportion of the incidence of severe anaemia up to the initial 12 weeks of treatment. The incidence of severe anaemia, (a) ≥3 g/dL reduction or (b) <10 g/dL of haemoglobin, was more frequent in the patients with CC at rs1127354 [65% (145/224), 33% (73/224)] than in those with CA/AA [25% (21/85), 6% (8/85)] ($P < 0.0001$).

Association between ITPA genetic variants and anti-HCV treatment outcomes or RBV adherence

We examined the pretreatment factors associated with SVR. By univariate analysis, gender, age, baseline Hb, platelet count, gamma-glutamyl transpeptidase, HCV RNA level and rs8099917 and rs1127354 genotypes were significantly associated with SVR. In logistic regression models that included these factors, age, platelet count, gamma-glutamyl transpeptidase, HCV RNA level and rs8099917 and rs1127354 genotypes were independent pretreatment predictive factors for SVR (Table 3). *IL28B* genetic variants were very strongly associated with SVR ($P < 0.0001$, OR = 23.9); therefore, we analysed the associations between *ITPA* genetic variants and treatment outcome in subgroups according to *IL28B* genotype. In all patients, the proportions of SVR, TVR and NVR were 44, 25 and 31%, respectively. In patients with TT at rs8099917 (*IL28B* favourable type), the SVR rate in patients with CA/AA at rs1127354 was higher than in those with CC [72% (46/64) *vs* 55% (78/143), $P = 0.019$], while no difference in the SVR rate was found in the *IL28B* unfavourable type due to poor virologic response (Fig. 2). When we examined the administered dosage of RBV during treatment in patients with TT at rs8099917, by dividing into three groups; ≥80%, ≥60 to <80% and <60% of the planned RBV dosage, the proportion of patients administered ≥80% of the dosage of RBV was significantly higher in CA/AA than in CC at 1127354 ($P = 0.025$, Fig. 3), resulting in a lower relapse rate [19% (12/64) *vs* 34% (48/143)] (Fig. 2).

It has been reported that the incidence of RBV-induced anaemia was more frequent in older patients, resulting in poor adherence to RBV; therefore, we examined the associations between *ITPA* genetic variants and treatment outcome according to age in the patients with the *IL28B* favourable type. As a result, the SVR rate was higher in ≥60-year-old patients with CA/AA at rs1127354 than in those with CC [71% (22/31) *vs* 40% (26/65), $P = 0.005$], although there was no significant difference in treatment efficacy according to *ITPA* genetic variants in the <60-year-old patients (Fig. 4a). Regardless of age, the percentage of patients receiving ≥80% of the planned RBV dosage was higher in the patients with CA/AA at rs1127354 than in those with CC; however, the differences were not significant in <60- or ≥60-year-old patients (Fig. 4b). In this study, there were 46 patients aged ≥65 years with *IL28B* favourable type. In these patients, the dosage of RBV also tended to be higher in the patients with CA/AA at rs1127354 than in those with CC, resulting in higher SVR rate that in those with CA/AA [(9/14 (64%) *vs* 11/32 (34%)].

DISCUSSION

Haemolysis is a common side effect of RBV and is the major reason for the dose reduction in RBV. Age, female gender, baseline platelet level, baseline Hb level and the plasma concentration of RBV have been reported to contribute to RBV-induced anaemia and dose reduction [26–28]. In several countries, including Japan, administration of erythropoietin-stimulating agents to CHC patients during PEG-IFN plus RBV treatment is not approved, and so RBV-induced anaemia could influence the treatment response, especially the relapse rate.

Recently, *ITPA* genetic variants have been shown to predict RBV-induced anaemia in European American [19] and Japanese populations [20]. In addition, we have identified that *ITPA* genetic variants are associated with reduction in

Table 2 Univariate and multivariate regression analysis of pretreatment factors associated with Hb < 10 g/dL up to 12 weeks

	Univariate analysis			Multivariate analysis	
	Hb < 10 g/dL (n = 78)	Hb ≥ 10 g/dL (n = 231)	P value	OR (95% CI)	P value
Male gender	24	136	<0.0001	-	-
Age, years	61 ± 8	55 ± 10	<0.0001	1.04 (1.01–1.09)	0.017
Body weight, kg	56 ± 10	61 ± 11	<0.001	-	-
Haemoglobin, g/dL	13.2 ± 1.4	14.4 ± 1.2	<0.0001	0.45 (0.41–0.73)	<0.0001
Platelet count, $\times 10^4$ /μL	15.4 ± 5.2	16.7 ± 5.7	0.079		
ALT, IU/L	62 ± 47	79 ± 69	0.056		
Creatinine, mg/dL	0.7 ± 0.2	0.7 ± 0.2	0.550		
rs1127354, CC/CA+AA	73/5	151/80	<0.0001	7.73 (2.87–20.82)	<0.0001

Data are expressed as number for categorical data or the mean ± standard deviation for continuous data. rs1127354: CA/AA is protectable to RBV-induced anaemia in PEG-IFN plus RBV therapy.

Table 3 Univariate and Multivariate regression analysis of pretreatment factors associated with sustained virologic response

	Univariate analysis			Multivariate analysis	
	SVR (n = 136)	non-SVR (n = 173)	P value	OR (95% CI)	P value
Male gender	79	81	0.049	-	-
Age, years	55 ± 10	58 ± 10	0.004	0.96 (0.93–01.00)	0.028
Body weight, kg	61 ± 11	59 ± 11	0.116		
Hb, g/dL	14.3 ± 1.2	13.9 ± 1.4	0.008	-	-
Platelet count, $\times 10^4$ /μL	17.9 ± 6.1	15.1 ± 4.9	<0.0001	1.10 (1.03–1.16)	0.004
ALT, IU/L	79 ± 69	71 ± 61	0.299		
γ-GTP, IU/L	47 ± 55	71 ± 80	0.003	0.99 (0.99–1.00)	0.012
HCV RNA, log IU/mL	6.1 ± 0.6	6.3 ± 0.5	<0.0001	0.27 (0.14–0.51)	<0.0001
rs8099917, TT/TG+GG	124/12	83/90	<0.0001	24.52 (9.94–60.48)	<0.0001
rs1127354, CC/CA+AA	87/49	137/6	0.003	2.57 (1.29–5.13)	0.008

Data are expressed as number for categorical data or the mean ± standard deviation for continuous data. rs8099917: TT is favourable to treatment efficacy, and rs1127354: CA/AA is protectable to RBV-induced anaemia in PEG-IFN plus RBV therapy.

platelet counts as well as anaemia in PEG-IFN plus RBV therapy [29]. The *ITPA* genetic variation causing an accumulation of inosine triphosphate (ITP) has been shown to protect patients against RBV-induced anaemia during PEG-IFN plus RBV treatment. It has previously been reported that the two functional variants in the *ITPA* gene, rs1127354 in exon 2 and rs7270101 in intron 2, were associated with ITPase deficiency, resulting in an accumulation of ITP in erythrocytes [30–33]. A recent report showed a biologic mechanism in which ITP confers protection against RBV-induced ATP reduction by substituting for erythrocyte GTP, which is depleted by RBV, in the biosynthesis of ATP [34].

The previous studies examined the association between *ITPA* genetic variants and RBV-induced anaemia at week 4 after the start of PEG-IFN plus RBV therapy [19–21]. However, it is important to evaluate the incidence of severe anaemia, which requires reduction in RBV dosage, which usually occurs up to week 12 after the start of therapy. In the present study, we replicated the previous study that *ITPA* genetic variants were associated with Hb reduction during PEG-IFN plus RBV treatment. Furthermore, we indicated that the incidence of severe anaemia during the initial 12 weeks of treatment was more frequent in patients with CC at rs1127354 than in those with CA/AA. In addition, *ITPA* genotype, pretreatment Hb levels and age were independent predictive factors for severe anaemia (Hb < 10 g/dL) up to week 12 by multivariate logistic regression analysis. In older patients, pretreatment Hb levels tend to be lower than in younger patients, suggesting

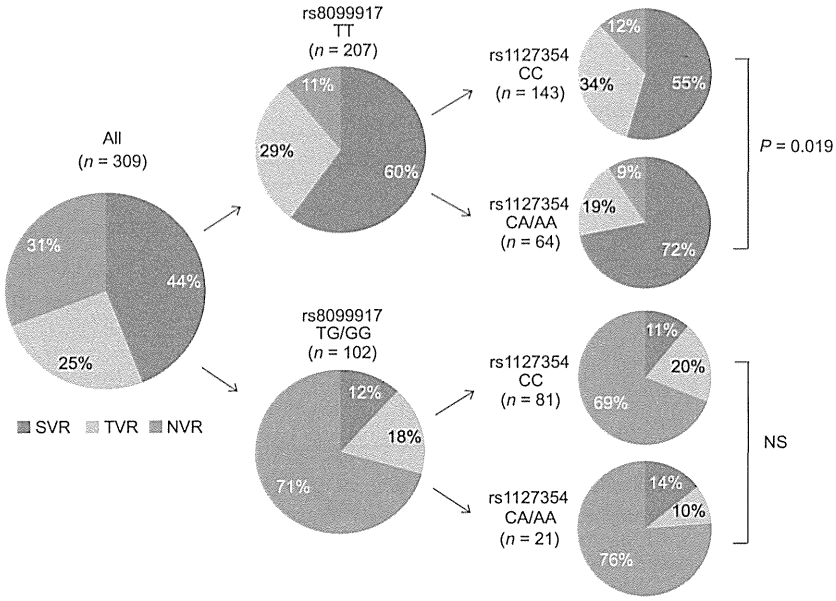

Fig. 2 Treatment outcome according to *IL28B* and *ITPA* genetic variants. In patients with *IL28B* favourable type, rs8099917: TT, the sustained viral response rate in the patients with CA/AA at rs1127354, protected against RBV-induced anaemia, was higher than in those with CC [72% (46/64) *vs* 55% (78/143), $P = 0.019$], while no difference in the sustained viral response rate was found in the *IL28B* unfavourable type (rs8099917: TG/GG) due to poor virologic response. SVR, sustained viral response; TVR, transient viral response; NVR, null viral response.

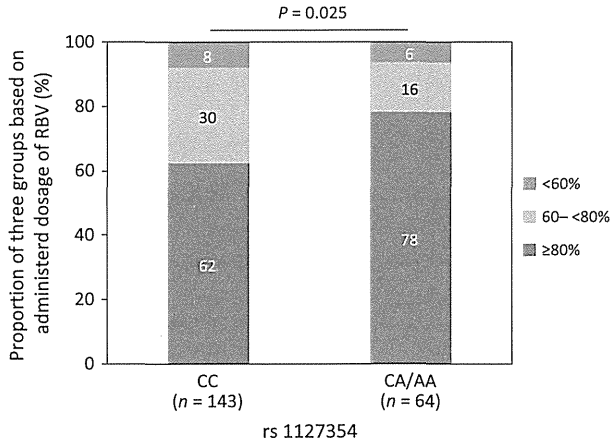

Fig. 3 Proportions of administered dosage of ribavirin in patients with *IL28B* favourable type. We examined the administered dosage of ribavirin during treatment in patients with *IL28B* favourable type, rs8099917: TT, by dividing them into three groups: ≥80%, ≥60 to <80% and <60% of the planned ribavirin dosage. The proportion of patients administered ≥80% of the dosage of ribavirin was significantly higher in the patients with CA/AA, protected against RBV-induced anaemia, than in those with CC at 1127354 ($P = 0.025$).

that not only *ITPA* genotype, but also age is an important predictive factor causing severe anaemia. In Japan, older HCV-infected patients developing liver fibrosis have been prevalent [11]; therefore, *ITPA* genotype is of significance in the Japanese population consisting of many elderly CHC patients.

With regard to the relationship between the effect of treatment and *ITPA* genetic variants, no association between the *ITPA* genetic variants and SVR was demonstrated in two previous studies of genotype 1 CHC patients [19, 24]. However, some reports from Japan suggested an association between *ITPA* genetic variants and SVR [21, 22], and Kurosaki *et al.* reported that the protective variant of *ITPA* was associated with less reduction in RBV and a high SVR rate in patients with *IL28B* favourable type [23]. In this study, the *ITPA* genetic variants were associated with treatment outcome in the overall cohort, especially in the patients with *IL28B* favourable type; the SVR rate in patients with CA/AA at rs1127354 was higher than in those with CC. The percentage of patients receiving ≥80% of the planned RBV dosage was higher in patients with CA/AA at rs1127354 than in those with CC. It has been reported that reduction in RBV to <80% results in a decreased rate of SVR [7]. Taken together, we showed that *ITPA* genetic variants were associated with the dose of RBV and the relapse rate in patients with *IL28B* favourable type.

It is well known that treatment efficacy is poorer in elderly patients, and the incidence of drug dose reduction or discontinuation could increase according to old age as well as advanced stage [10, 11]; however, we showed that the SVR rate and dosage of RBV in ≥60-year-old patients

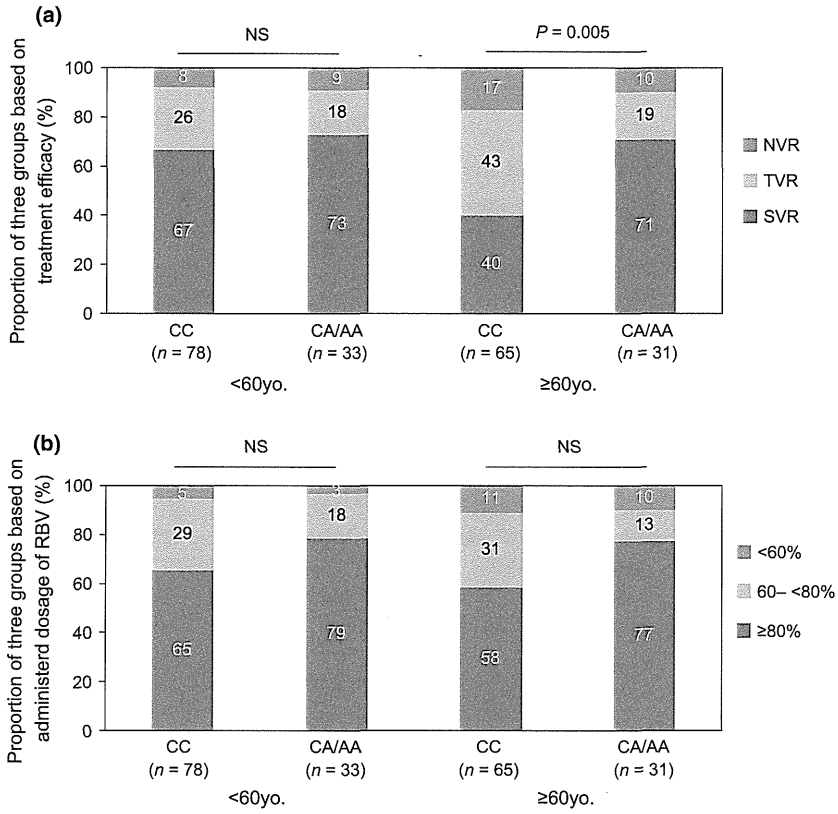

Fig. 4 Treatment outcome and administered dosage of ribavirin according to age and *ITPA* genetic variants in patients with *IL28B* favourable type. (a) The sustained viral response rate was higher in ≥60-year-old patients with CA/AA at rs1127354, protected against RBV-induced anaemia, than in those with CC [71% (22/31) *vs* 40% (26/65), $P = 0.005$], although there was no significant difference in treatment efficacy according to *ITPA* genetic variants in the <60-year-old patients. SVR, sustained viral response; TVR, transient viral response; NVR, null viral response. (b) Regardless of age, the percentage of patients receiving ≥80% of the planned RBV dosage was higher in the patients with CA/AA at rs1127354 than in those with CC; however, the differences were not significant in <60- or ≥60-year-old patients.

with CA/AA at rs1127354 were equivalent to <60-year-old patients (Fig. 4). In Japan, HCV-infected patients are relatively older than in Europe and the USA. In the present study, the risk of severe anaemia was higher, and treatment efficacy was poorer in elderly patients with CC at rs1127354. Additionally, administration of erythropoietin is not allowed in Japan; therefore, *ITPA* genetic variants could influence the treatment efficacy more strongly in the Japanese CHC population than in European and American populations. In these patients with a high risk of severe anaemia, it is necessary for erythropoietin to be used to improve treatment efficacy.

Recently, direct-acting antiviral agents (DAAs), such as telaprevir, have been shown to have a strong antiviral effect on HCV; however, treatment outcome was poorer in IFN and telaprevir without than in IFN and telaprevir with RBV regimens in clinical trials; thus, RBV is a key drug for treatment efficacy in regimens including IFN and DAAs as well as PEG-IFN/RBV therapy. In regimens including PEG-IFN, RBV and telaprevir, severe anaemia is a serious adverse effect [9, 35]. Recently, it was reported that *ITPA* genetic variants influenced haemoglobin levels during telaprevir, PEG-IFN plus RBV therapy [36]; however, the association between *ITPA* genotype and treatment efficacy did not reach significance [37]. In intractable cases with *IL28B* unfavourable type, RBV adherence could influence treatment response [38]; therefore, *ITPA* genetic variants associated with RBV adherence might be important for achieving SVR by triple combination therapy. As more recently reported, the *ITPA* SNP (rs1127354) may be a useful tool in predicting patients susceptible to RBV-induced anaemia during IFN-free treatments for HCV [39].

In conclusion, *ITPA* genetic variants, pretreatment Hb levels and age were associated with severe RBV-induced anaemia. In addition, *ITPA* genetic variants could influence the efficacy of PEG-IFN plus RBV treatment among elderly patients with *IL28B* favourable type. These findings may

have the potential to support an individualized treatment strategy.

ACKNOWLEDGEMENTS

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CONFLICT AND INTERESTS

Yasuhito Tanaka has carried out contracted research for MSD (Merck Sharp & Dohme) and Chugai Pharmaceutical Co. Ltd.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Reduction in haemoglobin levels up to the initial 12 weeks of treatment according to *ITPA* genetic variants.

Fig. S2. Proportion of haemoglobin <10 g/dL up to the initial 12 weeks of treatment according to baseline haemoglobin level, age and *ITPA* genetic variants.

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DEEP SEQUENCING ANALYSIS OF VARIANTS RESISTANT TO THE NS5A INHIBITOR DACLATASVIR IN PATIENTS WITH GENOTYPE 1B HEPATITIS C VIRUS INFECTION.

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Short title: Deep sequencing for daclatasvir-resistant HCV.

Abbreviations:

HCV: hepatitis C virus, IFN: interferon, PEG: pegylated, RBV: ribavirin, SVR: sustained virological response, TPV: telaprevir, BPV: boceprevir, DAA: direct antiviral

agent, ISDR: interferon sensitivity-determining region, IRRDR: interferon-ribavirin resistance determining region, NS3: non-structural protein 3, NS5A: non-structural protein 5A, NS5B: non-structural protein 5B, SNP: single nucleotide polymorphism, IL28B: interleukin 28B.

FOOTNOTES

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ABSTRACT

Background & Aims: Daclatasvir, an NS5A replication complex inhibitor, is a potent and promising direct antiviral agent (DAA) for hepatitis C virus (HCV), being most effective in genotype 1b infection. Although it is known that genotype 1b viruses with Y93H and/or L31M/V/F mutations have strong resistance to daclatasvir, it is not known whether there are some clinical background conditions that favor the occurrence of HCVs carrying those NS5A mutations

Methods: In this study, we carried out deep sequencing analysis of stored sera to determine the presence and significance of daclatasvir-resistant mutants in 110 genotype 1b HCV-infected patients with no previous daclatasvir treatment.

Results: Deep sequencing analysis revealed that the NS5A L31M/V/F and Y93H mutations were present in 13/110 (11.8%) and 34/110 (30.9%) patients, respectively, and significantly more frequently than in the control plasmid. Simultaneous L31M/V/F and Y93H mutations were detected in 4/110 patients (3.6%). When the clinical relevance of NS5A resistance was investigated, Y93H was significantly correlated with the IL28B major (TT) genotype of the host ($p = 0.042$).

Conclusions: Y93H was detected frequently by deep sequencing in daclatasvir treatment-naïve patients. Importantly, it seems that the IL28B status of the patients might influence the presence of Y93H mutations, resulting in different treatment responses to daclatasvir.

Key words: HCV, deep sequencing, NS5A inhibitor, resistance

INTRODUCTION

Recently, treatment of hepatitis C virus (HCV) infection has advanced markedly. Specifically, the advent of telaprevir (TPV) and boceprevir (BPV), first-generation protease inhibitors, dramatically increased the sustained virological response (SVR) rate to as high as 60% to 80% by combination with pegylated (PEG)-interferon (IFN)/ribavirin (RBV) therapy [1]. However, high SVR rates following combination therapy have not been seen in null-responders to previous PEG-IFN/RBV combination therapy [2]. Under these circumstances, development of more effective drug therapies with less serious adverse effects is anticipated.

Daclatasvir (BMS-790052), a nonstructural (NS) 5A replication complex inhibitor, is a potent and promising direct antiviral agent (DAA) for HCV. Daclatasvir has anti-HCV activity with broad genotypic coverage, but is most effective for genotype-1b viruses [3]. Moreover, among all NS5A inhibitors, daclatasvir is most advanced in its development for clinical use [4, 5]. Drug-resistant mutations have been identified for daclatasvir, and resistance is acquired by Y93H, L31M/V/F or P32L substitutions in NS5A in genotype 1b HCV. In particular, simultaneous substitutions of Y93H and L31M/V/F produce more robust resistance [6, 7].

In Japan, a clinical phase II trial of 24-week combination therapy of two oral agents, the NS5A inhibitor daclatasvir and NS3 protease inhibitor asunaprevir (BMS-650032), was carried out in 43 patients with genotype 1b HCV infection. The therapy achieved an SVR rate of 90.5% in patients with a null-response to PEG-IFN/RBV combination therapy and of 63.6% in patients considered ineligible or intolerant to IFN-based therapy [8, 9]. The result was that the SVR rate was markedly high, in particular, in patients with a null-response to PEG-IFN/RBV combination

therapy, giving hope to these difficult-to-treat patients. The study also revealed that the presence of Y93H prior to treatment was significantly associated with non-SVR to the regimen of the two oral agents [8-11]. On the other hand, it remains unknown whether differences in clinical backgrounds, including previous history of IFN therapy and its response, are associated with the presence of Y93H in daclatasvir-treatment naïve genotype 1b patients

In this study, we carried out deep sequencing analysis using a second generation sequencer to determine the presence of daclatasvir-resistant viruses in genotype 1b HCV patients. By deep sequencing, viral mutants associated with DAA resistance and present as minor populations could be detected [12-14]. Because daclatasvir is considered to be a key DAA for therapy for HCV in the near-future, we tried to clarify the possible clinical significance of HCV resistance mutations, such as Y93H, in the treatment response and their possible association with other viral and host factors.

PATIENTS AND METHODS

Patients

The subjects were 110 randomly-selected, daclatasvir treatment-naïve patients who were infected with genotype 1b HCV and followed-up at the Yamanashi University Hospital. The 110 patients included 59 naïve patients, 30 relapser patients (defined as patients with reappearance of HCV RNA after the completion of previous PEG-IFN/RBV combination therapy carried out between 2005 and 2011) and 21 null responder patients (defined as patients without a 2 log drop of HCV RNA at week 12 compared to that at week 0 during previous PEG-IFN/RBV combination therapy carried out between 2005 and 2011). These three groups of patients with distinctly different treatment responses to previous therapy (naïve, relapse, and null) were included in this study to clarify whether the rate of NS5A mutations varies among different backgrounds of the treatment response. None of the 51 patients who had failed to eradicate the virus during PEG-IFN/RBV combination therapy had received antiviral therapy thereafter. In the 110 patients, daclatasvir resistance mutations were analyzed by deep sequencing of sera collected and stored at the most recent visit to the hospital.

All patients studied fulfilled following criteria: (1) Negative for hepatitis B surface antigen. (2) No other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease. (3) Free of co-infection with human immunodeficiency virus. (4) Signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital. The clinical backgrounds of the 110 patients are shown in Table 1.

Direct sequencing

HCV RNA extraction, complementary DNA synthesis, amplification by two-step nested PCR from serum samples using primers specific for partial viral regions and direct sequencing were carried out as described previously [15, 16]. Generated sequence files were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram.

This direct sequencing procedure was performed to determine the dominant viral sequences of the core [17], the interferon sensitivity-determining region (ISDR) [18] and the interferon-ribavirin resistance determining region (IRRDR) [19] from the serum of each patient.

IL28B SNP analysis

Recent reports have disclosed a significant correlation between polymorphisms in the interleukin (IL) 28B gene and patients' responses to pegylated-IFN plus ribavirin therapy for HCV [20-22]. Human genomic DNA was extracted from peripheral blood using a blood DNA extraction kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol. The genotyping of each DNA sample was performed by real-time PCR with a model 7500 sequencer (ABI, Tokyo, Japan) using FAM- and VIC-labeled single nucleotide polymorphism (SNP) probes for the locus rs8099917 (ABI).

Deep sequencing

Deep sequencing of part of the viral NS5A region was performed for each of the 110 patients. Briefly, RNA was extracted from the stored sera and reverse transcribed to complementary DNA [23]. Then, two-step nested PCR was carried out

with primers specific for the NS5A region of the HCV genome. To avoid PCR selection bias, we searched for the most conserved DNA sequence regions around NS5A by examining sequence information published previously from 43 HCV-positive individuals from Japan [16] and designed novel primers for this study (Supplementary Table1). This PCR procedure amplified 436 viral nucleotides, including the 1st to 432nd nucleotide of the NS5A region. The primers for the second-round PCR had barcodes, 10 nucleotides (nt) in length, attached and these differed for each sample, so that the PCR products from each sample were identifiable. After the band densities of the PCR products were quantified using a Pico Green® dsDNA Assay Kit (Invitrogen™), the concentrations of the samples were adjusted to a common value and pooled samples were prepared.

Libraries were then subjected to emulsion PCR, the enriched DNA beads were loaded onto a picotiter plate and pyrosequencing was carried out with a Roche GS Junior/454 sequencing system using titanium chemistry (Roche, Branford, CT). The Roche Variant Analyzer version 2.5pl (Roche) was used for the analysis.

Statistical Analysis

Statistical differences in the parameters, including all available patients' demographic, biochemical, hematological, virological, and SNP data in the three groups (naïve, relapser and null responder), classified according to the response to previous PEG-IFN/RBV therapy, were determined using the Chi-square test for categorical variables and Kruskal-Wallis test for numerical variables. Statistical differences in the parameters in two groups (Y93H positive, Y93H negative) were determined by the Student t test or Mann-Whitney's U test for numerical variables and Fisher's exact

probability test or Chi-square test for categorical variables. Variables that achieved statistical significance ($p < 0.05$) in univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. We also calculated the odds ratios and 95% confidence intervals. All p values of <0.05 by the two-tailed test were considered significant.

RESULTS

Average read numbers obtained by deep sequencing and the background error rate

To perform deep sequencing analysis of the NS5A region from many patients, simultaneous analysis was carried out using the barcode primers and approximately 3826 reads were obtained per sample from each group of patients (naïve, relapser and null responder) (Table 2). Because a previous clinical phase 2 study had yielded a significantly high SVR rate, especially in the patients with a null response to previous PEG-IFN/RBV combination therapy, we classified the patients according to their responses to previous PEG-IFN/RBV combination therapy with the assumption that differences in the response to PEG-IFN/RBV might influence the daclatasvir response.

The background error rate of pyrosequencing was calculated with a plasmid containing a cloned HCV sequence (pCV-J4L6S) [24] and the read number for the plasmid is also shown in Table 2. Though seven runs of the plasmid produced 2,277-7,000 reads, with an average of 5,448 reads, there was no background error at amino acid (aa) 31, 32 or 93 in NS5A. Because the background error rate was 0% at each position, the presence of mutations at 0.1% or higher was considered to be significant, based on the 95% confidence interval (0 - 0.1%) calculated for 0% in 2,227 reads. The background error rate coincided almost exactly with the background error rate obtained in our recent study [23].

Baseline characteristics

The baseline characteristics of the 110 patients are shown in Table 1. The data for viral factors (core aa 70, core aa 91, NS5A-ISDR and NS5A-IRRDR) in the table were obtained by direct sequencing as described in ~~the~~ Patients and Methods. As shown

in the table, there were significant differences among the three groups in AST, ALT, γGTP, alpha-fetoprotein, core aa 70, and IL28B SNP (rs8099917). Meanwhile, there was no significant difference in background factors of age and gender or liver fibrosis associated factors such as PLT and Alb.

Detection of NS5A resistance mutations by deep sequencing

Because previous reports showed that L31M/V/F, P32L, and Y93H are resistance mutations in NS5A of genotype 1b HCV, the presence of these mutations was analyzed by deep sequencing. Table 3 shows the rate of NS5A resistance mutations at aa 31, 32, and 93. At aa 32, no mutation was fond in any of the 110 patients. Regarding aa 31, resistance mutations (L31M/V/F) were observed in 13/110 patients (11.8%) and, despite no significant difference, tended to occur more frequently in the relapser group and naïve group than in the null group. Meanwhile, the aa 93 resistance mutation (Y93H) was observed in 34/110 (30.9%) and, despite no significant difference, also tended occur more frequently in the naïve group and relapser group than in the null group. Simultaneous aa 93 and 31 resistance mutations were observed in only 4/110 patients (3.6%) and these four patients all belonged to the naïve group. More detailed deep sequence results for the four patients with simultaneous mutation of L31M/V/F and Y93H are shown in Supplementary Table 2. Although the substitution rate of L31M/V/F in these patients was low, all isolates with L31M/V/F also featured the Y93H change.

Mutation rates of L31M/V/F and Y93H in each patient

Figure 1A and B show the mutation rates of L31M/V/F and Y93H in each

patient. One bar indicates the resistance mutation rate in one patient, obtained by deep sequencing. It was found that minor viral populations that were not detected by direct sequencing could be detected by deep sequencing.

In order to compare our deep sequencing data with previous direct sequencing data in terms of the frequency of NS5A mutations, the notion of "cut-offs" was introduced into our deep sequencing data, assuming that direct sequencing could detect minor populations existing above those cut-off levels. When the cut-off level of 50% was defined to detect minor populations by direct sequencing, L31M/V/F mutations and the Y93H mutations were detected in 1.8% (2/110 patients) and 7.3% (8/110) of our patients, respectively, while the values became 1.8% (2/110 patients) and 15.4% (15/110) when 20% was defined as the cut-off level. These results are comparable to the mutation rate determined previously by direct sequencing and that found in the database [25].

Univariate and multivariate analysis of factors related to the NS5A Y93H mutation

Focusing on the Y93H mutation that is found most frequently in daclatasvir-treatment naïve patients, clinical background factors that would determine efficacy of PEG-IFN/RBV combination therapy patients were investigated by univariate analysis of their association with the Y93H substitution (Table 4). Three factors, the IL28B SNP, core aa 70, and IRRDR, were found to be correlated with the Y93H substitution with statistical significance in the univariate analysis. In patients with the Y93H mutation, the major-type (TT) was frequently observed as the IL28B SNP, while arginine (R) was frequently observed at core aa 70 and the number of substitutions in the IRRDR was higher. There was no significant difference in the number of mutations

in the ISDR but that number tended to be higher in patients with the Y93H mutation, similar to the IRRDR.

The IL28B SNP, core aa 70, and IRRDR, which were correlated significantly with the aa 93 mutation by univariate analysis, were subjected to multivariate analysis (Table 4). The IL28B SNP major-type (TT) was extracted as an independent significant factor with the odds ratio of 3.67 ($p = 0.042$). The mutation rates of L31M/V/F and Y93H in each patient, classified by the IL28 SNP, are presented in Figure 2A and B. Y93H mutations were found significantly more frequently in IL28B TT patients than that in IL28B non-TT patients.